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(73) Proprietor: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES
10010 North Torrey Pines Road
La Jolla California 92037 (US)

(72) Inventor: ESCH, Frederick, Stephen
2929 Fire Mountain Drive, 63
Oceanside, CA 92507 (US)
Inventor: BAIRD, Andrew
7102 Teasdale
San Diego, CA.92122 (US)
Inventor: BOHLEN, Peter
Huhnliackerweg 3
CH-8610 Uster (CH)
Inventor: GOSPODAROWICZ, Denis, Jersy
215 Maywood Drive
San Francisco, CA 94127 (US)
Inventor: LING, Nicholas, Chai-Kwan
5324 Bloch Street
San Diego, CA 92122 (US)

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(74) Representative: Lawrence, Malcolm Graham et al
Hepworth, Lawrence & Bryer
2nd Floor, Gate Hous South
Westgate Road
Harlow Essex CM20 1JN (GB)

Description

The present invention is directed to a DNA encoding basic polypeptides active as a fibroblast growth factor (bFGF). The invention relates also to bFGF polypeptides demonstrating both high purity (about 98% by weight of total proteins and greater) and FGF activity. The invention further provides synthetic methods, which will substantially enhance the availability of mammalian FGF.

Both the brain and the pituitary gland have been known to contain mitogenic factors for cultured cells; however, until 1974, it was unclear what their relationship was with classical pituitary hormones, such as TSH, LH, FSH, GH and ACTH. In 1974, the identification in the pituitary gland of a growth factor called fibroblast growth factor (FGF) was reported which was shown to be distinct from pituitary hormones. Gospodarowicz, D. Nature, 249, 123-127 (1974). This growth factor is now known to have a MW of 16,415, is basic (as pI of 9.6), and is a potent mitogen for either normal diploid fibroblasts or established cell lines. Purification of an acidic brain FGF is described in US Patent No 4,444,760 (Apr. 24, 1984). Later studies confirmed that, in addition to fibroblasts, FGF is also mitogenic for a wide variety of normal diploid mesoderm-derived and neural crest-derived cells, including granulocytes, adrenal cortical cells, chondrocytes, myoblasts, corneal and vascular endothelial cells from either bovine or human origin, vascular smooth muscle cells, and lens epithelial cells. FGF was also shown to substitute for platelet-derived growth factor in its ability to support the proliferation of fibroblasts exposed to plasma-supplemented medium. Consistent with its ability to stimulate the proliferation of bovine and vascular endothelial cells, FGF has a similar activity in vivo on capillary endothelial cells; therefore, FGF is considered an angiogenic factor.

Mammalian fibroblast growth factor (FGF) can be purified using reverse-phase high performance liquid chromatography (RP-HPLC) and by the use of heparin-Sepharose affinity chromatography. Such methods of purifying FGF from mammalian tissue, such as brain and/or pituitary tissue, may be difficult to scale up to large scale production, and accordingly the production of pure FGF by synthetic methods should substantially enhance the availability of mammalian FGF.

Proc Natl Acad Sci. Vol 81, 5364-68, 1984 discloses a technique for isolation in alleged homogenous form of relatively small quantities of a partially characterized growth factor from bovine pituitaries, the factor having a MW of 16000 and an amino terminal 1-15 sequence as noted in Formula I set forth hereinafter. Purity of 90% or more is claimed but the RHPLC technique used to achieve this destroys 90-95% of the biological activity of the factor. Proc Natl Acad Sci. Vol 81, 6963-67, 1984 discloses a technique for isolation in relatively small quantities and in alleged homogenous form of a pituitary growth factor functioning as a basic mitogen and composed of single polypeptide chains of MW 14000 - 16000. Use of improved procedures is claimed to achieve 90% a higher purity with recovery of about 80% of biological activity. Biochem Biophys Res Comm, Vol 128, 554-62, 1985 discloses isolation of a human placental fibroblast growth factor found by the authors to be very similar to the amino acid composition of bovine pituitary FGF and found also to stimulate the proliferation of the same cell types that respond to bovine FGF.

The present invention provides pure basic 146-amino acid residue fibroblast growth factor (bFGF) as defined below and a method by which it may be synthesized using recombinant DNA techniques or other suitable techniques. By bFGF is meant a 146 amino acid residue polypeptide having the sequence set forth hereinafter or an equivalent thereof. It appears most likely that in the native molecule none of the cysteine residues are disulfide bonded to each other, but that there may be bonding of one (or more) of the cysteine residues to another free cysteine molecule. Evidence for there being no internal disulfide-bonding between cysteine residues is in fact not fully conclusive and it may be that one or two pairs of cysteine residues are internally bonded to each other. In any case, the present invention provides biologically active peptides, whether non-bonded or randomly bonded. Because bFGF is a relatively long-chain peptide, synthesis by a recombinant DNA technique is the synthetic method of choice, as opposed to standard chain elongation procedures involving stepwise addition of amino acid residues. Extraction and purification are possible but are not considered to be commercially feasible at the present time. Accordingly, a bFGF-encoding DNA chain is obtained, eg by oligonucleotide synthesis, and the synthetic DNA chain is inserted into a cloning vector, appropriately placed therein so as to ensure its expression when the recombinant cloning vector is introduced into an organism or cell line. Synthetic bFGF polypeptides which either have no internal disulfide bonds or which are randomly disulfide bonded exhibit biological activity.

Pharmaceutical compositions in accordance with invention include bFGF, a bFGF analog, biologically active fragments of bFGF or of analog bFGF, or nontoxic salts thereof, dispersed in a pharmaceutically acceptable liquid or solid carrier. Such pharmaceutical compositions can be used in clinical medicine, both human and veterinary, in acuter or chronic administration for diagnostic or therapeutic purposes. bFGF is further useful in in vitro cell proliferation procedures. Also considered to be within the scope of the invention are peptides with additional segments added to either or both termini, such as those which arise from

considerations of vector construction when the peptides are made using recombinant DNA techniques, providing that such terminal segments do not destroy the biological activity of the peptide.

The invention provides the first known pure (ie 98% purity or more based on total protein) and active mammalian bFGF, and the production ther of by synthetic methods. The nomenclature used to define the peptides is that specified by Schroder & Lubke, "The Peptides", Academic Press (1965), wher in in accordance with conventional representation the r sidue having the free alpha-amino group at the N-terminus appears to left and the residue having the alpha-carboxyl group at the C-terminus to the right. Where the amino acid r sidue has isomeric forms, it is the L-form of the amino acid that is represented. The bFGF peptides of the invention, when of 146-residue length, has the Formula I noted below:

```
     1   2   3   4   5   6   7   8   9  10  11  12  13  14  15
H-Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-

    16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-

    31  32  33  34  35  36  37  38  39  40  41  42  43  44  45
Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-

    46  47  48  49  50  51  52  53  54  55  56  57  58  59  60
Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-

    61  62  63  64  65  66  67  68  69  70  71  72  73  74  75
Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-

    76  77  78  79  80  81  82  83  84  85  86  87  88  89  90
Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-

    91  92  93  94  95  96  97  98  99 100 101 102 103 104 105
Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-

   106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-

   121 122 123 124 125 126 127 128 129 130 131 132 133 134 135
Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-

   136 137 138 139 140 141 142 143 144 145 146
Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser-Y,
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wherein Y is OH or NH_2. It is uncertain whether the C-terminus of the native molecule is amidated. For purposes of this application, bFGF peptides should be considered to constitute peptides having the 146 amino-acid-residue sequence as well as biologically active fragments thereof.

Included within the scope of the invention is DNA encoded for the 146-amino acid residue, an example of DNA according to the invention being the synthetic gene of Example 2 hereinafter.

From presently available evidence, it is most likely that there is no internal disulfide-bonding between cysteine residues of the chain, as noted earlier herein. Two of the cysteine residues may be internally disulfide-bonded to each other, and the residues at positions 25 and 69 are likely candidates for internal bonding; however, it appears that this is in fact unlikely One or more of the cysteine residues, excluding any which are involved in internal disulfide bonding, may be bonded to free cysteine. The invention is intended to encompass synthetically produced bFGF polypeptides in which the cysteines are free or have random internal disulfide bonds, ie, between positions 25 and 69; 25 and 87; 25 and 92; 69 and 87; 69 and 92; 87 and 92; 25 and 69 plus 87 and 92; 25 and 87 plus 69 and 92; and 25 and 92 plus 69 and 87. A mixture of FGF peptides in which cysteine residues are non-bonded or randomly bonded exhibits at least some biological activity bFGF or "basic FGF" has a basic pI of 9.6 (in contrast to acidic FGF which has an acidic

pI of about 5).

In any event, bFGF polypeptides produced by recombinant DNA techniques are inherently biologically active. This may be because the three-dimensional structure which bFGF assumes within cells is the structure recognized by the receptor. The thr e-dimensional structure which the molecule assumes through natural folding and through hydrophobic and hydrophilic interactions with aqueous media may promote desired bonding or non-bonding between cysteine r sidues. Also, enzymatic r gulatory mechanisms within cells may help to ensure desired disulfide bonding or non-bonding, either by preventing bonding or by directing disulfide bonding between particular cysteine residues. Enzymes might also cleave "incorrect" bonding to enable the molecule to reorientate itself and assume the correct natural structure. Cysteine residues that are not internally bonded may be disulfide-bonded to free cysteine moieties. It may also be that the three-dimensional structure of the molecule is such that random bonding or non-bonding of cysteine residues either with each other or to free cysteines does not substantially affect the biological structure of the protein molecule.

To synthesize a protein having the bFGF amino acid residue sequence by recombinant DNA, a double-stranded DNA chain which encodes bFGF is synthetically constructed. The segment of the DNA chain that encodes bFGF is, of course, designed according to the genetic code; however, because of the degeneracy of the genetic code, a wide variety of codon combinations can be selected to form the DNA chain that encodes the product polypeptide. It is known that certain particular codons are more efficient for polypeptide expression in certain types of organisms, and the selection of codons preferably is made according to those codons which are most efficient for expression in the type of organism which is to serve as the host for the recombinant vector. However, any correct set of codons will encode product, even if slightly less efficiently. Codon selection may also depend upon vector construction considerations: for example, it may be necessary to avoid placing a restriction site in the DNA chain if, subsequent to inserting the synthetic DNA chain, the vector is to be manipulated using the restriction enzyme that cleaves at such a site. Also, it is necessary to avoid placing restriction sites in the DNA chain if the host organism which is to be transformed with the recombinant vector containing the DNA chain is known to produce a restriction enzyme that would cleave within the DNA chain.

In addition to the bFGF-encoding sequences, the DNA chain that is synthesized may contain additional sequences, depending upon vector construction considerations. Typically, the DNA chain is synthesized with linkers at its ends to facilitate insertion into restriction sites within a cloning vector. The DNA chain may be constructed so as to encode the bFGF amino acid sequences as a portion of a fusion polypeptide; and if so, it will generally contain terminal sequences that encode amino acid residue sequences that serve as proteolytic processing sites, whereby the bFGF polypeptide may be proteolytically cleaved from the remainder of the fusion polypeptide. The terminal portions of the synthetic DNA chain may also contain appropriate start and stop signals.

To assemble a bFGF-encoding DNA chain, oligonucleotides are constructed by conventional methods, such as procedures described in T. Manatis et al., Cold Spring Harbor Laboratory Manual, Cold Spring Harbor, New York (1982)(hereinafter, CSH). Sense and antisense oligonucleotide chains, up to about 70 nucleotide residues long, are synthesized, preferably on automated synthesizers, such as the Applied Biosystem Inc. model 380A DNA synthesizer. The oligonucleotide chains are constructed so that portions of the sense and antisense oligonucleotides overlap, associating with each other through hydrogen binding between complementary base pairs and thereby forming double stranded chains, in most cases with gaps in the strands. Subsequently, the gaps in the strands are filled in and oligonucleotides of each strand are joined end to end with nucleotide triphosphates in the presence of appropriate DNA polymerases and/or with ligases.

As an alternative to construction of a synthetic DNA chain through oligonucleotide synthesis, cDNA corresponding to bFGF may be prepared. A cDNA library or an expression library is produced in a conventional manner by reverse transcription from messenger RNA (mRNA) from a bFGF-producing cell line. To select clones containing bFGF sequences, hybridization probes (preferably mixed probes to accommodate the degeneracy of the genetic code) corresponding to portions of the FGF protein are produced and used to identify clones containing such sequences. Screening of the expression library with FGF antibodies may also be used, alone or in conjunction with hybridization probing, to identify or confirm the presence of bFGF-encoding DNA sequences in DNA library clones. Such techniques are taught, for example in CSH, supra.

The double-stranded bFGF-encoding DNA chain is constructed or modified with insertion into a particular appropriate cloning vector in mind. The cloning vector that is to be recombined to incorporate the DNA chain is selected appropriate to its viability and expression in a host organism or cell line, and the manner of insertion of the DNA chain depends upon factors particular to the host. For example, if the DNA

chain is to be inserted into a vector for insertion into a prokaryotic cell, such as E. Coli, the DNA chain will be inserted 3' of a promoter sequence, a Shine-Delgarno sequence (or ribosome binding site) that is within a 5' non-translated portion and an ATG start codon. The ATG start codon is appropriately spaced from the Shine-Delgarno sequence, and the encoding sequence is placed in correct reading frame with the ATG start codon. The cloning vector also provides a 3' non-translated region and a translation termination site. For insertion into a eukaryotic cell, such as a yeast cell or a cell line obtained from a higher animal, the bFGF-encoding oligonucleotide sequence is appropriately spaced from a capping site and in correct reading frame with an ATG start signal. The cloning vector also provides a 3' non-translated region and a translation termination site.

Prokaryotic transformation vectors, such as pBR322, pMB9, Col EI, pCRI, RP4 and lambda-phage, are available for inserting a DNA chain of the length which encodes bFGF with substantial assurance of at least some expression of the encoded polypeptide. Typically, such vectors are constructed or modified to have a unique restriction site(s) appropriately positioned relative to a promoter, such as the lac promoter. The DNA chain may be inserted with appropriate linkers into such a restriction site, with substantial assurance of production of bFGF in a prokaryotic cell line transformed with the recombinant vector. To assure proper reading frame, linkers of various lengths may be provided at the ends of the bFGF-encoding sequences. Alternatively, cassettes, which include sequences, such as the 5' region of the lac Z gene (including the operator, promoter, transcription start site, Shine Delgarno sequence and translation initiation signal), the regulatory region from the tryptophane gene (trp operator, promoter, ribosome binding site and translation initiator), and a fusion gene containing these two promoters called the trp-lac or commonly called the Tac promoter are available into which the synthetic DNA chain may be conveniently inserted and then the cassette inserted into a cloning vector of choice.

Similarly, eukaryotic transformation vectors, such as, the cloned bovine papilloma virus genome, the cloned genomes of the murine retroviruses, and eukaryotic cassettes, such as the pSV-2 gpt system (described by Mulligan and Berg, Nature 277, 108-114, 1979) the Okayama-Berg cloning system (Mol. Cell Biol. 2, 161-170, 1982), the expression cloning vector recently described by Genetics Institute (Science 228, 810-815, 1985), are available which provide substantial assurance of at least some expression of bFGF in the transformed eukaryotic cell line.

A convenient way to ensure production of FGF or a polypeptide of a similar length is to produce the polypeptide initally as a segment of a gene-encoded fusion polypeptide. In such case, the DNA chain is constructed so that the expressed polypeptide has enzymatic processing sites flanking the bFGF amino acid residue sequences. A bFGF-encoding DNA chain may be inserted, for example, into the beta-galactosidase gene for insertion into E. Coli, in which case, the expressed fusion polypeptide is subsequently cleaved with proteolytic enzymes to release the bFGF from beta-galactosidase peptide sequences.

An advantage of inserting the bFGF-encoding sequence so that the bFGF sequence is expressed as a cleavable segment of a fusion polypeptide, e.g., as the bFGF peptide sequence fused within the beta-galactosidase peptide sequence, is that the endogenous polypeptide into which the bFGF sequence is inserted is generally rendered non-functional, thereby facilitating selection for vectors encoding the fusion peptide.

Example 1

The structure of basic FGF of the bovine species was determined as follows:

Frozen bovine pituitaries were homogenized with a Waring blender for 5 minutes in 0.15 M ammonium sulfate (4 liter kg tissue). The pH was then adjusted to 4.5 with HCl and the homogenate stirred vigorously for 2 hours. After centrifugation (18,000 x g, 30 minutes) the supernatant was retained, and 230g ammonium sulfate per liter of supernatant were added; the pH was adjusted to 6-6.5 with NaOH; and the precipitation was allowed to proceed for 15 hours. After centrifugation of the reaction mixture (18,000 x g, 30 min), the supernatant was retained; 300g ammonium sulfate were added to each liter of the supernatant, and then the mixture stirred well for two hours. After centrifugation of the reaction mixture (18,000 x g, 30 min), the pellet was retained, and the cumulative pellets from 3 kg starting tissue was dissolved in 200 ml distilled water and dialyzed against 20 liters of distilled water overnight. The pH of the dialyzed retentate was then adjusted to 6, and the solution was clarified by centrifugation (12,000 x g, 30 min). The dialyzed retentate consititutes a dialyzed extract.

Basic FGF was subsequently isolated from the dialyzed, clarified extract using three successive protocols; two of these employed conventional ion-exchange and reverse phase HPLC purification steps as described previously (P. Bohlen et al. Proc. Natl. Acad. Sci. USA 81, 5364-5368 (1984)). The third method utilized heparin-Sepharose affinity chromatography in a key purification step as detailed as follows in the

order in which they were performed.

(A) CM-Sephadex (C50) ion-exchange chromatography.

A 7 x 9 cm column of carboxymethyl Sephadex (C50) was washed with 1 liter of 50 mM sodium phosphate, 1.5 M sodium chloride, pH 6.0 and then equilibrated with 0.1 M sodium phosphate, pH 6.0. The dialyzed extract from 3 kg bovine pituitaries was loaded onto the column, and the column was washed s quentially with 0.1 M sodium phosphate, pH 6.0 containing a) no NaCl, b) 0.2 M NaCl and c) 0.65 M NaCl, allowing the OD_{280} to reach a minimum value before initiating each new wash. Fractions of 18 ml were collected at 3 ml·min at 4 °C and subjected to radioimmunoassay.

(B) Heparin-Sepharose chromatography.

The 0.65 M NaCl eluate from CM-Sephadex chromatography was loaded onto a 3 X 3 cm column of heparin-Sepharose (Pharmacia) previously equilibrated with 10 mM Tris-HCl, 0.6 M NaCl, pH 7.0 at room temperature. The column was then washed sequentially with 10 mM Tris-HCl, pH 7.0 containing a) 0.6 M NaCl and b) 1.1 M NaCl, allowing the OD_{280} to reach a minimum value with each wash. The basic FGF was then eluted with a linear gradient in 10 mM Tris-HCl, pH 7.0 containing 100 ml 1.1 M NaCl and 100 ml 2 M NaCl. Fractions of 5 ml were collected at 0.8 ml/min and subjected to radioimmunoassay.

(C) Reverse phase liquid chromatography.

The basic FGF from heparin-Sepharose chromatography was pumped onto a Vydac C-4 (0.46 X 25 cm) reverse phase column (The Separations Group, Inc.) using a 0.1% trifluoroacetic acid (TFA)/acetonitrile solvent system (F. S. Esch et al. Methods in Enzymol. (ed. Conn, P.) 103, Academic Press, NY, pp. 72-89 (1983)) and eluted at 0.6 ml/min, with a 90 min. gradient of 23% to 35% acetonitrile. Fractions of 3 ml were collected at room temperature and subjected to radioimmunoassay.

In the above mentioned Radioimmunoassays (RIA) for basic FGF, antibodies were generated against a synthetic analog of the amino terminal sequence of basic FGF, [Tyr^{10}]FGF(1-10) which is conjugated to bovine serum albumin, and were subsequently used to develop the radioimmunoassay for basic FGF, as described in A. Baird et al. Regulatory Peptides 10, 309-317 (1985).

Because it is not possible to quantitate unmodified cysteine by amino acid analysis, cysteine residues were modified either by reduction and alkylation with [^{14}C]iodoacetamide (New England Nuclear) or oxidization with performic acid as indicated below. In either case, the FGF in 0.1% TFA/acetonitrile was dried in a 1.5 ml polypropylene microfuge tube in a Speed Vac vacuum centrifuge (Savant, Inc.) just prior to modification.

The reduction and alkylation of cysteine residues was performed in order to radioactively label cysteine residues, making it possible to determine which fragments of subsequent cleavage reactions contain cysteine residues. The dried bFGF was dissolved in 0.1 ml deoxygenated 0.5M Tris-HCl pH 7.7, 10mM EDTA, 6M guanidine-HCl. Dithiothreitol was added to a final concentration of 5-10 mM, and the reduction was allowed to proceed at 37 °C for 30 min. A 0.5-fold molar excess of [^{14}C]iodoacetamide (24 mCi/mmole) over total sulfhydryl groups was added, and the incubation continued at 37 °C for 60 min. in the dark. The alkylation was terminated by addition of a large excess of dithiothreitol over iodoacetamide, and the alkylated FGF was purified by reverse phase-high performance liquid chromatography.

Performic acid oxidation of cysteine convers cysteine to cysteic acid: and the cysteic acid content of the protein is measurable by amino acid analysis. Performic acid was generated by incubating 9 ml distilled formic acid with 1 ml 30% H_2O_2 at room temperature in a tightly capped tube for 1 hour. 0.25 ml of this solution was employed to dissolve the dried FGF (5-15 nmoles), and the oxidation was permitted to continue at 0 °C for 2.5 hours. Four lyophilizations from distilled water were employed to remove reaction by-products.

Basic FGFs (with cysteines modified by each method described above) were proteolytically and chemically digested to obtain fragments for further analysis, including sequence analysis. Prior to any digestion the FGF was dried in a polypropylene microfuge tube in a Speed Vac vacuum centrifuge from volatile RP-HPLC solvents.

In order to obtain multiple, overlapping FGF fragments, three types of proteolytic digestions of bFGFs, with cysteines modified by each method described above, were performed as follows. The dried FGF (1-5 nmoles) was dissolved in 0.01 ml 0.5 M Tris-HCl pH 7.7, 10 mM EDTA, 6 M guanidine-HCl and then diluted to 1 ml with 1% NH_4HCO_3. Submaxillaris protease or chymotrypsin was added in a 1/50 (w/w) ratio while digestions with Staphylococcus aureus V8 employed a 1:35 (mol:mol) ratio of enzyme to substrate. Submaxillaris protease cleaves at the C-terminus of arginine: Staphylococcus aureus V8 cleaves at the C-terminus of glutamic acid: and chymotrypsin cleaves at the C-terminus of several amino acid residues having bulky aromatic or hydrophobic groups. Incubations were allowed to proceed overnight at 37 °C.

Digestion with cyanogen bromide, which cleaves proteins at the C-terminus of Met, were performed on bFGFs, with cysteines modified by each method described above, as follows. The dried, alkylated FGF (5-6

nmoles) was dissolved with 0.05 ml 70% formic acid and reduced in a solution of 2.9 M N-methylmercaptoacetamide in 7% formic acid (R. Houghten et al. Methods in Enzymol. (eds. Hirs., C. & Timasheff, S.) 91. Academic Press, NY. pp. 549-559 (1983)) for 24 hours at 37°C. The alkylated, reduced FGF was purified by RP-HPLC, dried in a Speed Vac vacuum centrifuge and redissolved in 0.1 ml deoxygenated 70% formic acid. A 100-fold excess of cyanogen bromide was added and the incubation continued at room temperature in the dark overnight.

Reverse phase-high performance liquid chromatography purifications of modified bFGFs and their digestion fragments were accomplished using a Brownlee RP-300 reverse phase column (0.46 x 25 cm) and a 0.1% TFA:acetonitrile or a 0.1% heptafluorobutyric acid (HFBA)-acetonitrile solvent system (Esch et al. (1983) supra.).

Amino acid analyses and gas phase micro-sequencing of intact bFGF and its digestion fragments were carried out by methods previously described (P. Bohlen et al. Anal. Biochem. 126, 144-152 (1982); F. S. Esch Anal. Biochem. 136, 39-47 (1984)). PhNCS-(^{14}C)-carboxyamidomethylcysteine was identified during sequence analysis by liquid scintillation counting of the residues from the sequencer. The identification of cysteic acid in a given cycle was accomplished by comparison of the amino acid composition of the peptide and the remainder of its sequence as determined by Edman degradation. Carboxypeptidase Y was obtained from Pierce and utilized according to the manufacturer's recommendations. Carboxyl terminal analysis via tritium incorporation was accomplished as previously described (H. Matsuo et al. Protein Sequence Determination (ed., Needleman, S.B.) Springer-Verlag, NY. pp. 104-113 (1979)).

The highly efficient purification procedure, described above, permitted the rapid isolation of large quantities (about 30 to 60 nmoles per week) of highly purified basic FGF from bovine pituitaries. This source aided in the structural characterization effort. The heparin-Sepharose affinity chromatography purification step resulted in a several thousand-fold purification of two biologically active and basic FGF-immunoreactive mitogens, eluting at approximately 1.4 M and 1.85 M NaCl. A single step of RP-HPLC effected peptide homogeneity in each case. NaDodSO$_4$ PAGE yielded identical molecular weight estimates for both species, and gas phase micro-sequencing showed that both possessed identical amino terminal amino acid sequences through at least the amino-terminal 24 residues of each polypeptide. Pituitary extracts yielded approximately 15 times more of the mitogen eluting at 1.4 M NaCl than of the later eluting species, and hence, the former was selected for further structural characterization.

NaDodSO$_4$ PAGE suggested a molecular weight of 16,250 ± 1000 for bovine pituitary basic FGF. Table I below shows the amino acid compositions obtained for the cationic mitogen from bovine brain and hypothalamus by R. R. Lobb et al., Biochem. 23, 6295-6299 (1984) as well as the compositional data obtained for basic FGF from bovine pituitary, all data being normalized for a 146 amino acid structure. The similarity of the compositions suggests that these structures are closely related, if not identical. In fact, basic FGF from bovine brain has been isolated, and it has been determined that its amino terminal sequence is identical to that of the pituitary-derived molecule

TABLE 1

Amino Acid compositions of basic FGF from different bovine tissue				
Amino Acid	Brain[a]	Hypothalamus[a]	Pituitary Basic FGF[b]	(1-146)
Asx	13.7	13.0	12.4 ± 0.4	12
Thr	5.1	4.9	3.9 ± 0.3	4
Ser	10.0	10.0	9.4 ± 0.6	10
Glx	13.2	14.2	14.1 ± 0.4[c]	12
Pro	11.6	11.3	9.4 ± 0.6	10
Gly	17.3	18.2	16.6 ± 0.6[c]	15
Ala	9.1	9.0	9.5 ± 0.4	9
Cys	n.d.	n.d.	4.3 ± 0.2[d]	4
Val	5.8	5.7	5.9 ± 0.7	7
Met	2.4	2.4	1.6 ± 0.4	2
Ile	3.2	3.1	3.4 ± 0.5	4
Leu	12.6	12.9	13.4 ± 0.4	13
Tyr	6.5	6.2	6.8 ± 0.4	7
Phe	7.9	7.6	7.5 ± 0.2	8
His	3.2	3.2	2.4 ± 0.6	3
Lys	13.7	13.5	13.9 ± 0.7	14
Arg	10.8	10.4	11.6 ± 0.3	11
Trp	n.d.	n.d.	0.4 ± 0.2	1

a Data from Lobb et al. supra. normalized for 146 amino acids.
b Amino acid composition of basic FGF deduced from sequence analysis.
c Discrepancy between amino acid and sequence analysis data greater than that expected from statistical analysis.
d Cysteine was determined as cysteic acid after RP-HPLC purification of performic acid oxidized basic FGF.

Example 2

Using conventional methods, described in CSH, supra., a synthetic bFGF gene is constructed having the following formula:

```
5' AATTCATGCCAGCCCTACCAGAAGATGGGGGGTCCGGGGCCTTCCCACCAGGG
3'     GTACGGTCGGGATGGTCTTCTACCCCCCAGGCCCCGGAAGGGTGGTCCC

      CACTTCAAAGATCCAAAACGACTATATTGTAAAAACGGGGGGTTC
      GTGAAGTTTCTAGGTTTTGCTGATATAACATTTTTGCCCCCCAAG

      TTCCTACGAATCCACCCAGATGGGCGAGTAGATGGGGTACGAGAA
      AAGGATGCTTAGGTGGGTCTACCCGCTCATCTACCCCATGCTCTT

      AAATCCGATCCACACATCAAACTACAACTACAAGCCGAAGAACGA
      TTTAGGCTAGGTGTGTAGTTTGATGTTGATGTTCGGCTTCTTGCT

      GGGGTAGTATCCATCAAAGGGGTATGTGCCAACCGATATCTAGCC
      CCCCATCATAGGTAGTTTCCCCATACACGGTTGGCTATAGATCGG

      ATGAAAGAAGATGGGCGACTACTAGCCTCCAAATGTGTAACCGAT
      TACTTTCTTCTACCCGCTGATGATCGGAGGTTTACACATTGGCTA

      GAATGTTTCTTCTTCGAACGACTAGAATCCAACAACTATAACACC
      CTTACAAAGAAGAAGCTTGCTGATCTTAGGTTGTTGATATTGTGG

      TATCGATCCCGAAAATATTCCTCCTGGTATGTAGCCCTAAAACGA
      ATAGCTAGGGCTTTTATAAGGAGGACCATACATCGGGATTTTGCT

      ACCGGGCAATATAAACTAGGGCCAAAAACCGGGCCAGGGCAAAAA
      TGGCCCGTTATATTTGATCCCGGTTTTTGGCCCGGTCCCGTTTTT

      GCCATCCTATTCCTACCAATGTCCGCCAAATCCTAAG      3'
      CGGTAGGATAAGGATGGTTACAGGCGGTTTAGGATTCAGCT  5'
```

Synthesis of a bFGF-encoding DNA chain is accomplished by synthesizing oligonucleotides on an applied B10 systems automatic synthesizer with overlapping complementary sequences.

The overlapping oligonucleotides are fused to form a double-stranded DNA chain, gaps being filled in with DNA polymorase and with T4 ligase. Immediately 5' of the FGF-encoding sequence in the sense strand is provided an ATG start signal, which results in an extraneous methionine being added to the N-terminus of the expressed polypeptide. Immediately 3' of the bFGF-encoding sequence is a stop signal. At the 5' end is a Eco RI overhang and at the 3' end is a Sal I overhang, whereby the synthetic DNA strand is directly insertable in the Eco RI and Sal I site of the plasmid pUC8, described by Vieira er al. Gene 14, 259-268 (1982) The DNA strand is annealed into the pUC8 plasmid where it is under the control of the beta galactosidase promoter with the ATG start signal and the Shine Delgarno sequence retained in their natural orientation and association with the promoter.

The recombinant vector, designated bFGF, is transformed into the DH-I strain of E. Coli by the calcium chloride procedure, CSH, supra.

The transformed E. Coli is cultured in L broth, and ampicillan-resistant strains are selected. Because the DNA chain was inserted into the plasmid in an orientation which could be expected to lead to expression of protein product of the DNA chain, the ampicillan-resistant colonies are screened for reactivity with antiserum raised against bFGF extracted from the pituitary These colonies are screened by the immunological method of Healfman et al . Proc Natl. Acad. Sci. USA 80, 31-35 (1983), and colonies reacting positively with bFGF antibody are further characterized. The cells are separated from their culture media are lysed, and their supernatent obtained Supernatent from transformed cells is determined by RIA to be reactive with antibody raised against bFGF

100 ml of cell supernatent is obtained, and bFGF is purified therefrom using heparin-Sepharose as described above Approximately 0.01 mg. of FGF, purified to upwards of 98% by weight of total protein, is produced

The biological activity of the synthetic bFGF, which contains the extraneous N-terminal methionine residue, is tested for biological activity by the ability of the synthetic bFGF to stimulate the proliferation of adult bovine aortic arch endothelial cells in culture, as described in J. Cell Biol. 97, 1677-1685 (1983).

Briefly, cells (at passage 3-10) are seeded at a density of 2×10^3 cells/dish on plastic tissue culture dishes and exposed to Dulbecco's modified Eagle's m dium (DMEM) supplemented with 10% calf serum. Test samples, at a dilution ranging from 10^{-1} to 10^{-3}, are added on day 0 and day 2 to the dishes. On day 4, triplicate dishes are trypsinized and counted in a Coulter counter. Background levels are ordinarily 10^5 cells/dish, while those exposed to optimal concentrations of the growth factor can contain as much as 5 to 8 $\times 10^5$ cells. For a potency assay, a log response curve was established. For this purpose, 10 microliter-aliquots of a dilution (ranging from 10^{-1} to 10^{-5}) of the original solution made in 0.5% bovine serum albumin (BSA)/DMEM were added in triplicate.

The biological (mitogenic) activity of synthetic bFGF is substantially identical to natural, purified bFGF.

The superfluous N-terminal residue is removable by partial chemical digestion with cyanogen bromide or phenyl isothiocyanate followed by treatment with a strong anhydrous acid, such as trifluoroacetic acid. However, this process attacks internal Met residues, and while providing some bFGF having the natural protein structure, substantially reduces the total amount of biologically active protein.

Example 3

Plasmid bFGF, amplified in one of the bFGF-producing E. Coli clones of Example 2, is isolated and cleaved with Eco RI and Sal I. This digested plasmid is electrophoresed on an agarose gel allowing for the separation and recovery of the amplified bFGF insert. The insert is inserted into the plasmic pYEp, a shuttle vector which can be used to transform both E. Coli and Saccharomyces cerevisiae yeast. Insertion of the synthetic DNA chain at this point assures that the DNA sequence is under the control of a promoter, in proper reading frame from an ATG signal and properly spaced relative to a cap site. The shuttle vector is used to transform URA3, a strain of S. cerevisiae yeast from which the oratate monophosphate decarboxylase gene is deleted.

The transformed yeast is grown in medium to attain log growth. The yeast is separated from its culture medium, and cell lysates are prepared. Pooled cell lysates are determined by RIA to be reactive with antibody raised against bFGF, demonstrating that a peptide containing bFGF peptide segments is expressed within the yeast cells.

The invention provides polypeptides and should make this important material available for biological and therapeutic use. The production of bFGF can be carried out in both prokaryotic and eukaryotic cell lines. While bFGF synthesis is easily demonstrated using either bacteria or yeast cell lines, the synthetic genes should be insertable for expression in cells of higher animals, such as mammalian tumor cells. Such mammalian cells may be grown, for example, as peritoneal tumors in host animals, and bFGF harvested from the peritoneal fluid.

Although the above examples demonstrate that bFGF can be synthesized through recombinant DNA techniques, the examples do not purport to have maximized bFGF production. It is expected that subsequent selection of more efficient cloning vectors and host cell lines will increase the yield of bFGF. Known gene amplification techniques for both eukaryotic and prokaryotic cells may be used to increase production of bFGF. Secretion of the gene-encoded polypeptide from the host cell line into the culture medium is also considered to be an important factor in obtaining synthetic FGF in large quantities.

FGF may also be synthesized using either classical synthesis and/or solid-phase synthesis to produce peptide segments of reasonable length. Such segments can then be appropriately linked to one another to create the desired 146-residue molecule.

Brain and pituitary FGF preparations, as reported earlier, are mitogenic for a wide variety of normal diploid cultured cells derived from tissue originating from the primary or secondary mesenchyme as well as from neuroectoderm. These include rabbit chondrocytes, bovine granulosa and adrenal cortex cells, bovine corneal endothelial cells, capillary endothelial cells derived from bovine adrenal cortex and human umbilical endothelial cells.

bFGF peptides are useful biological materials for promoting in vitro growth of cultured cell lines, such as cell lines that have been transformed by recombinant DNA techniques to produce other useful polypeptides.

Furthermore, studies have shown that bFGF is capable of eliciting an angionenic response, for example, when implanted in the hamster cheek pouch or in the chick chorioallantoic membrane. Accordingly, substantially pure bFGF peptides have potential therapeutic applications.

Substantially pure FGF polypeptides can be routinely obtained having significantly high r purity than FGF polypeptides that are extracted from mammalian tissues, such as bovine pituitaries. FGF polypeptides constitue only very minor constituents of normal mammalian tissues and thus are present only in v ry impure form, relative to other native polypeptides also present. Recombinant DNA techniques, for example,

can be used to generate organisms or cell lines that produce the heterologous polypeptide in significantly higher proportions relative to total protein, in the cellular material and/or their secretions, than the proportions at which native FGF polypeptides are present in mammalian tissue. Because the starting material from which such synthetic FGF polypeptides are isolated has a substantially greater concentration of the heterologous polypeptide, purification techniques can fairly simply produce more highly purified FGF polypeptide fractions. Using isolation techniques such as those described hereinbefore, it is possible to routinely obtain bFGF polypeptides which are at least about 98% pure (by weight of total proteins) and which is herein referred to as substantially pure.

Substantially pure synthetic bFGF or the nontoxic salts thereof, combined with a pharmaceutically acceptable carrier to form a pharmaceutical composition, may be administered to mammals, including humans, either intravenously, subcutaneously, intramuscularly or orally. The required dosage will vary with the particular condition being treated, with the severity of the condition and with the duration of desired treatment.

Such peptides are often administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g., with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder, such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate. If administration in liquid form is desired, sweetening and/or flavoring may be used, and intravenous administration in isotonic saline, phosphate buffer solutions or the like may be effected.

The peptides should be administered under the guidance of a physician, and pharmaceutical compositions will usually contain the peptide in conjunction with a conventional, pharmaceutically-acceptable carrier.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art. For example, biologically active fragments can be employed instead of the entire 146-residue peptide, e.g. bFGF (24-120)-OH and bFGF(20-110)-NH_2.

Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. DNA encoding the amino acid sequence of a mammalian basic fibroblast growth factor (bFGF) polypeptide containing the 146-amino acid residue sequence:-

```
 1   2   3   4   5   6   7   8   9  10  11  12  13  14  15
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-

16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-

31  32  33  34  35  36  37  38  39  40  41  42  43  44  45
Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-

46  47  48  49  50  51  52  53  54  55  56  57  58  59  60
Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-

61  62  63  64  65  66  67  68  69  70  71  72  73  74  75
Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-

76  77  78  79  80  81  82  83  84  85  86  87  88  89  90
Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-

 91  92  93  94  95  96  97  98  99 100 101 102 103 104 105
Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-

106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-

121 122 123 124 125 126 127 128 129 130 131 132 133 134 135
Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-

136 137 138 139 140 141 142 143 144 145 146
Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser
```

2. DNA encoding the amino acid residue sequence of a bFGF polypeptide containing a fragment of the 146-amino acid residue sequence set forth in Claim 1, said polypeptide being biologically active as a fibroblast growth factor.

3. DNA as claimed in Claim 2 wherein the fragment is bFGF(24-120).

4. DNA encoding the amino acid residue sequence of a bFGF polypeptide containing an equivalent or an analogue of the 146-amino acid residue sequence set forth in Claim 1, namely a sequence of amino acid residues which is substantially identical thereto and which renders the polypeptide biologically active as a fibroblast growth factor.

5. DNA as claimed in Claim 1 and having the nucleotide sequence:-

```
5'  AATTCATGCCAGCCCTACCAGAAGATGGGGGGTCCGGGGCCTTCCCACCAGGG
3'      GTACGGTCGGGATGGTCTTCTACCCCCCAGGCCCCGGAAGGGTGGTCCC

        CACTTCAAAGATCCAAAACGACTATATTGTAAAAACGGGGGGTTC
        GTGAAGTTTCTAGGTTTTGCTGATATAACATTTTTGCCCCCCAAG

        TTCCTACGAATCCACCCAGATGGGCGAGTAGATGGGGTACGAGAA
        AAGGATGCTTAGGTGGGTCTACCCGCTCATCTACCCCATGCTCTT

        AAATCCGATCCACACATCAAACTACAACTACAAGCCGAAGAACGA
        TTTAGGCTAGGTGTGTAGTTTGATGTTGATGTTCGGCTTCTTGCT

        GGGGTAGTATCCATCAAAGGGGTATGTGCCAACCGATATCTAGCC
        CCCCATCATAGGTAGTTTCCCCATACACGGTTGGCTATAGATCGG

        ATGAAAGAAGATGGGCGACTACTAGCCTCCAAATGTGTAACCGAT
        TACTTTCTTCTACCCGCTGATGATCGGAGGTTTACACATTGGCTA

        GAATGTTTCTTCTTCGAACGACTAGAATCCAACAACTATAACACC
        CTTACAAAGAAGAAGCTTGCTGATCTTAGGTTGTTGATATTGTGG

        TATCGATCCCGAAAATATTCCTCCTGGTATGTAGCCCTAAAACGA
        ATAGCTAGGGCTTTTATAAGGAGGACCATACATCGGGATTTTGCT

        ACCGGGCAATATAAACTAGGGCCAAAAACCGGGCCAGGGCAAAAA
        TGGCCCGTTATATTTGATCCCGGTTTTTGGCCCGGTCCCGTTTTT

        GCCATCCTATTCCTACCAATGTCCGCCAAATCCTAAG      3'
        CGGTAGGATAAGGATGGTTACAGGCGGTTTAGGATTCAGCT  5'
```

6. DNA encoding the amino acid residue sequence of a bFGF polypeptide which contains a terminally extended analogue of the 146-amino acid residue sequence set forth in Claim 1 and which has biological activity as a fibroblast growth factor.

7. DNA as claimed in Claim 6 wherein the terminally extended analogue is N-terminally extended.

8. DNA as claimed in Claim 7 wherein the N-terminally extended analogue is a methionine-N-terminally extended analogue

9. DNA encoding the amino acid residue sequence of a fusion polypeptide containing a bFGF polypeptide sequence as defined in any preceding claim as a cleavable segment.

10. DNA as claimed in Claim 9 and including terminal nucleotide sequences encoding amino acid residue sequences which serve as proteolytic processing sites enabling the sequence of the bFGF polypeptide to be proteolytically cleaved from the fusion polypeptide sequence by an enzyme

11. DNA as claimed in Claim 9 or Claim 10 and including terminal portions containing start and stop signals.

12. DNA as claimed in any preceding claim and including terminal linkers for facilitating insertion of the DNA into restriction sites within a cloning vector

13. A cloning or expression vector comprising a DNA sequenc as claimed in any preceding claim.

14. A microorganism transformed with a vector as claimed in Claim 13 and capable of expr ssing the encoded bFGF polypeptide.

15. A microorganism according to Claim 14 which is a strain of E.Coli or a strain of S.cer visiae y ast.

16. A method of producing a bFGF polypeptide, said polypeptide being one encoded by DNA as defined in any one of Claims 1 to 12, the method comprising obtaining DNA as claimed in any one of Claims 1 to 12 that encodes the amino acid sequence of said bFGF polypeptide, inserting said DNA into a cloning vector in proper relationship to DNA sequences which promote expression thereof, transforming an organism or cell line with said cloning vector having said inserted DNA sequence, culturing said transformed organism or cell line, and obtaining the polypeptide encoded by said DNA and produced thereby, the method optionally including one or more of the following optional steps:
(i) when the obtained polypeptide is a fusion polypeptide, a step of cleaving from said fusion polypeptide said bFGF polypeptide; and
(ii) a step of conversion of the polypeptide to non-toxic salt form.

17. A method as claimed in Claim 16 wherein said DNA is a chain which has been synthesized by stepwise construction of oligonucleotides with overlapping complementary sequences.

18. A method as claimed in Claim 16 wherein said DNA is a chain which has been obtained by producing a cDNA clone library from a mammalian bFGF polypeptide-producing cell line, determining a clone having said DNA by hybridization with a probe, and excising said DNA from said cDNA clone.

19. A method as claimed in Claim 18 wherein said organism is a prokaryote.

20. A method as claimed in Claim 18 wherein said organism or cell line is a eukaryote.

21. Use of DNA as claimed in any one of Claims 1 to 12 to produce (i) bFGF polypeptide encoded thereby, (ii) a bFGF polypeptide as a cleavage product from a fusion polypeptide encoded thereby or (iii) a non-toxic salt thereof.

22. A polypeptide which:
(i) has the amino acid residue sequence coded by DNA as claimed in any one of Claims 1 to 12; and
(ii) has a purity of about 98% by weight (of total proteins) or greater; and
(iii) demonstrates biological activity as a fibroblast growth factor.

23. A polypeptide as claimed in Claim 22 wherein two of the four cysteine residues in the sequence of amino acid residues are internally disulfide bonded to each other.

24. A mammalian bFGF polypeptide which is the fragment bFGF(24-120)-OH or the fragment bFGF(20-110)-NH_2 relative to the 146-amino acid sequence set forth in Claim 1.

25. A non-toxic salt of a polypeptide as claimed in Claim 22 or Claim 23.

26. A non-toxic salt of a polypeptide as claimed in Claim 24.

27. A pharmaceutical composition comprising a bFGF polypeptide as claimed in Claims 22 or Claim 23 or a non-toxic salt thereof, together with a carrier

28. A pharmaceutical composition comprising a bFGF polypeptide as claimed in Claim 24 or a non-toxic salt thereof, together with a carrier.

29. Use of a bFGF polypeptide as claimed in Claim 22 or Claim 23 or a non-toxic salt thereof for the preparation of a diagnostic or therapeutic drug.

30. Use of bFGF polypeptide as claimed in Claim 24 or a non-toxic salt thereof for the preparation of a diagnostic or therapeutic drug

31. A mammalian bFGF polypeptide as claimed in Claim 22 or Claim 23 or a non-toxic salt thereof for use as a fibroblast growth factor in therapy or diagnostics.

32. A mammalian bFGF polypeptide as claimed in Claim 24, or a non-toxic salt thereof for use as a fibroblast growth factor in therapy or diagnostics.

33. A method for in vitro growth of cultured cells wherein growth is promoted by provision to said cells of a bFGF polypeptide as claimed in Claim 22 or Claim 23 or Claim 24, or a non-toxic salt thereof.

Claims for the following Contracting State : AT

1. A process for preparing a DNA sequence which is a chain which has been synthesized by stepwise construction of oligonucleotides with overlapping complementary sequences or which has been obtained by producing a cDNA clone library from a mammalian bFGF polypeptide-producing cell line, determining a clone having said DNA sequence by hybridization with a probe, and excising said DNA sequence from said cDNA clone, said DNA encoding:-

(i) the amino acid sequence of a basic fibroblast growth factor (bFGF) polypeptide containing the 146-amino acid residue sequence:-

```
 1   2   3   4   5   6   7   8   9  10  11  12  13  14  15
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-

16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-

31  32  33  34  35  36  37  38  39  40  41  42  43  44  45
Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-

46  47  48  49  50  51  52  53  54  55  56  57  58  59  60
Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-

61  62  63  64  65  66  67  68  69  70  71  72  73  74  75
Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-

76  77  78  79  80  81  82  83  84  85  86  87  88  89  90
Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-

 91  92  93  94  95  96  97  98  99 100 101 102 103 104 105
Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-

106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-

121 122 123 124 125 126 127 128 129 130 131 132 133 134 135
Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-

136 137 138 139 140 141 142 143 144 145 146
Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser
```

or

(ii) the amino acid residue sequence of a bFGF polypeptide containing a fragment of the 146-amino acid residue sequence set forth above, said polypeptide being biologically active as a fibroblast growth factor, or

(iii) the amino acid residue sequence of a bFGF polypeptide containing an equivalent or an analogue of the 146-amino acid residue sequence set forth above, namely a sequence of amino acid residues which is substantially identical thereto and which renders the polypeptide biologically active as a fibroblast growth factor, or

(iv) the amino acid residue sequence of a bFGF polypeptide which contains a terminally extended analogue of the 146-amino acid residue sequence set forth above and which has biological activity as a fibroblast growth factor.

2. A process as claimed in Claim 1 wherein the DNA sequence is DNA having the nucleotide sequence:-

```
5'    AATTCATGCCAGCCCTACCAGAAGATGGGGGGTCCGGGGCCTTCCCACCAGGG
3'        GTACGGTCGGGATGGTCTTCTACCCCCCAGGCCCCGGAAGGGTGGTCCC

          CACTTCAAAGATCCAAAACGACTATATTGTAAAAACGGGGGGTTC
          GTGAAGTTTCTAGGTTTTGCTGATATAACATTTTTGCCCCCCAAG

          TTCCTACGAATCCACCCAGATGGGCGAGTAGATGGGGTACGAGAA
          AAGGATGCTTAGGTGGGTCTACCCGCTCATCTACCCCATGCTCTT

          AAATCCGATCCACACATCAAACTACAACTACAAGCCGAAGAACGA



     TTTAGGCTAGGTGTGTAGTTTGATGTTGATGTTCGGCTTCTTGCT

     GGGGTAGTATCCATCAAAGGGGTATGTGCCAACCGATATCTAGCC
     CCCCATCATAGGTAGTTTCCCCATACACGGTTGGCTATAGATCGG

     ATGAAAGAAGATGGGCGACTACTAGCCTCCAAATGTGTAACCGAT
     TACTTTCTTCTACCCGCTGATGATCGGAGGTTTACACATTGGCTA

     GAATGTTTCTTCTTCGAACGACTAGAATCCAACAACTATAACACC
     CTTACAAAGAAGAAGCTTGCTGATCTTAGGTTGTTGATATTGTGG

     TATCGATCCCGAAAATATTCCTCCTGGTATGTAGCCCTAAAACGA
     ATAGCTAGGGCTTTTATAAGGAGGACCATACATCGGGATTTTGCT

     ACCGGGCAATATAAACTAGGGCCAAAAACCGGGCCAGGGCAAAAA
     TGGCCCGTTATATTTGATCCCGGTTTTTGGCCCGGTCCCGTTTTT

     GCCATCCTATTCCTACCAATGTCCGCCAAATCCTAAG      3'
     CGGTAGGATAAGGATGGTTACAGGCGGTTTAGGATTCAGCT  5'
```

3. A process as claimed in Claim 1 wherein the terminally extended analogue is N-terminally extended

4. A process as claimed in Claim 3 wherein the N-terminally extended analogue is a methionine-N-terminally extended analogue.

5. A process as claimed in Claim 1 wherein the DNA encodes the amino acid residue sequence of a fusion polypeptide containing a bFGF polypeptide sequence as defined in Claim 1 as a cleavable segment.

6. A process as claimed in Claim 5 wherein the DNA includes terminal nucleotide sequences encoding amino acid residue sequences which serve as proteolytic processing sites enabling the sequence of the bFGF polypeptide to be proteolytically cleaved from the fusion polypeptide sequence by an enzyme.

7. A process as claimed in Claim 5 or Claim 6 wherein the DNA includes terminal portions containing start and stop signals.

8. A process as claimed in any preceding claim wherein the DNA includes terminal linkers for facilitating insertion of the DNA into restriction sites within a cloning vector.

9. A method of producing a bFGF polypeptide, said peptide being one encoded by DNA as defined in any preceding claim, the method comprising obtaining DNA as defined in any preceding claim that encodes the amino acid sequence of said bFGF polypeptide, inserting said DNA into a cloning vector in proper relationship to DNA sequences which promote expression thereof, transforming an organism or cell line with said cloning vector having said inserted DNA sequence, culturing said transformed organism or cell line, and obtaining the polypeptide encoded by said DNA and produced thereby, the method optionally including one or more of the following optional steps:-
(i) when the obtained polypeptide is a fusion polypeptide, a step of cleaving from said fusion polypeptide said bFGF polypeptide; and
(ii) a step of conversion of the polypeptide to non-toxic salt form.

10. Use of DNA as defined in any one of Claims 1 to 8 to produce the bFGF polypeptide encoded thereby.

Patentansprüche
Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. DNA kodierend die Aminosäuresequenz eines Säuger-Basisfibroblasten-Wachstumsfaktor (bFGF) Polypeptides, enthaltend die 146-Aminosäure-Sequenz:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pro-	Ala-	Leu-	Pro-	Glu-	Asp-	Gly-	Gly-	Ser-	Gly-	Ala-	Phe-	Pro-	Pro-	Gly-
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
His-	Phe-	Lys-	Asp-	Pro-	Lys-	Arg-	Leu-	Tyr-	Cys-	Lys-	Asn-	Gly-	Gly-	Phe-
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Phe-	Leu-	Arg-	Ile-	His-	Pro-	Asp-	Gly-	Arg-	Val-	Asp-	Gly-	Val-	Arg-	Glu-
46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Lys-	Ser-	Asp-	Pro-	His-	Ile-	Lys-	Leu-	Gln-	Leu-	Gln-	Ala-	Glu-	Glu-	Arg-
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Gly-	Val-	Val-	Ser-	Ile-	Lys-	Gly-	Val-	Cys-	Ala-	Asn-	Arg-	Tyr-	Leu-	Ala-
76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Met-	Lys-	Glu-	Asp-	Gly-	Arg-	Leu-	Leu-	Ala-	Ser-	Lys-	Cys-	Val-	Thr-	Asp-
91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Glu-	Cys-	Phe-	Phe-	Phe-	Glu-	Arg-	Leu-	Glu-	Ser-	Asn-	Asn-	Tyr-	Asn-	Thr-
106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Tyr-	Arg-	Ser-	Arg-	Lys-	Tyr-	Ser-	Ser-	Trp-	Tyr-	Val-	Ala-	Leu-	Lys-	Arg-
121	122	123	124	125	126	127	128	129	130	131	132	133	134	135
Thr-	Gly-	Gln-	Tyr-	Lys-	Leu-	Gly-	Pro-	Lys-	Thr-	Gly-	Pro-	Gly-	Gln-	Lys-
136	137	138	139	140	141	142	143	144	145	146				
Ala-	Ile-	Leu-	Phe-	Leu-	Pro-	Met-	Ser-	Ala-	Lys-	Ser	.			

7. A process as claimed in Claim 5 or Claim 6 wherein the DNA includes terminal portions containing start and stop signals.

8. A process as claimed in any preceding claim wherein the DNA includes terminal linkers for facilitating insertion of the DNA into restriction sites within a cloning vector.

9. A method of producing a bFGF polypeptid , said peptide being one encoded by DNA as defined in any preceding claim, the method comprising obtaining DNA as defined in any preceding claim that encodes the amino acid sequence of said bFGF polypeptide, inserting said DNA into a cloning vector in proper relationship to DNA sequences which promote expression thereof, transforming an organism or cell line with said cloning vector having said inserted DNA sequence, culturing said transformed organism or cell line, and obtaining the polypeptide encoded by said DNA and produced thereby, the method optionally including one or more of the following optional steps:-

(i) when the obtained polypeptide is a fusion polypeptide, a step of cleaving from said fusion polypeptide said bFGF polypeptide; and

(ii) a step of conversion of the polypeptide to non-toxic salt form.

10. Use of DNA as defined in any one of Claims 1 to 8 to produce the bFGF polypeptide encoded thereby.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. DNA kodierend die Aminosäuresequenz eines Säuger-Basisfibroblasten-Wachstumsfaktor (bFGF) Polypeptides, enthaltend die 146-Aminosäure-Sequenz:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pro-	Ala-	Leu-	Pro-	Glu-	Asp-	Gly-	Gly-	Ser-	Gly-	Ala-	Phe-	Pro-	Pro-	Gly-
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
His-	Phe-	Lys-	Asp-	Pro-	Lys-	Arg-	Leu-	Tyr-	Cys-	Lys-	Asn-	Gly-	Gly-	Phe-
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Phe-	Leu-	Arg-	Ile-	His-	Pro-	Asp-	Gly-	Arg-	Val-	Asp-	Gly-	Val-	Arg-	Glu-
46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Lys-	Ser-	Asp-	Pro-	His-	Ile-	Lys-	Leu-	Gln-	Leu-	Gln-	Ala-	Glu-	Glu-	Arg-
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Gly-	Val-	Val-	Ser-	Ile-	Lys-	Gly-	Val-	Cys-	Ala-	Asn-	Arg-	Tyr-	Leu-	Ala-
76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Met-	Lys-	Glu-	Asp-	Gly-	Arg-	Leu-	Leu-	Ala-	Ser-	Lys-	Cys-	Val-	Thr-	Asp-
91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Glu-	Cys-	Phe-	Phe-	Phe-	Glu-	Arg-	Leu-	Glu-	Ser-	Asn-	Asn-	Tyr-	Asn-	Thr-
106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Tyr-	Arg-	Ser-	Arg-	Lys-	Tyr-	Ser-	Ser-	Trp-	Tyr-	Val-	Ala-	Leu-	Lys-	Arg-
121	122	123	124	125	126	127	128	129	130	131	132	133	134	135
Thr-	Gly-	Gln-	Tyr-	Lys-	Leu-	Gly-	Pro-	Lys-	Thr-	Gly-	Pro-	Gly-	Gln-	Lys-
136	137	138	139	140	141	142	143	144	145	146				
Ala-	Ile-	Leu-	Phe-	Leu-	Pro-	Met-	Ser-	Ala-	Lys-	Ser	.			

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Claims:

1. DNA encoding the amino acid sequence of a mammalian basic fibroblast growth factor (bFGF) polypeptide comprising the 146-amino acid residue sequence:

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 1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-

 18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34
Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-

 35  36  37  38  39  40  41  42  43  44  45  46  47  48  49  50  51
His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-

 52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67  68
Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-

 69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85
Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-

 86  87  88  89  90  91  92  93  94  95  96  97  98  99 100 101 102
Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119
Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-

120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-

137 138 139 140 141 142 143 144 145 146
Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,
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or the amino acid sequence in which 1 to 3 amino acids may be substituted in said amino acid sequence and which is biologically active as a fibroblast growth factor.

2. DNA according to Claim 1 encoding the 146-amino acid residue sequence:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-

18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-

35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51
His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-

52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68
Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-

69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85
Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-

86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102
Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119
Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-

120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-

137 138 139 140 141 142 143 144 145 146
Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,

or the amino acid sequence in which 1 to 3 amino acids may be substituted in the region of residues 1-19 and/or the region of residues 111-146 in said amino acid sequence.

3. DNA encoding the amino acid residue sequence of a bFGF polypeptide which comprises an N-terminally extended analogue of the 146-amino acid residue sequence:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-

18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-

35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51
His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-

52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68
Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-

69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85
Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-

86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102
Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119
Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-

120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-

137 138 139 140 141 142 143 144 145 146
Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,

and which has biological activity as a fibroblast growth factor.

4. DNA encoding an N-terminally shortened fragment of the 146 amino acid reside sequence:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-

18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-

35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51
His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-

52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68
Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-

69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85
Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-

86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102
Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119
Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-

120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-

137 138 139 140 141 142 143 144 145 146
Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,

which is biologically active as a fibroblast growth factor.

5. A cloning or expression vector comprising a DNA sequence selected from the group consisting of:
(1) DNA encoding the amino acid sequence of a mammalian basic fibroblast growth factor (bFGF) polypeptide comprising the 146-amino acid residue sequence:

```
 1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-

 18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34
Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-

 35  36  37  38  39  40  41  42  43  44  45  46  47  48  49  50  51
His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-

 52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67  68
Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-

 69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85
Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-

 86  87  88  89  90  91  92  93  94  95  96  97  98  99 100 101 102
Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119
Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-

120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-

137 138 139 140 141 142 143 144 145 146
Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,
```

or the amino acid sequence in which 1 to 3 amino acids may be substituted in said amino acid sequence and which is biologically active as a fibroblast growth factor;
(2) DNA encoding the amino acid residue sequence of a bFGF polypeptide which comprises an N-terminally extended analogue of the above 146-amino acid residue sequence and which is biologically active as a fibroblast growth factor; and

(3) DNA encoding an N-terminally shortened fragment of the above 146 amino acid reside sequence and which is biologically active as a fibroblast growth factor.

6. Bacteria or yeast transformed with a cloning or expression vector comprising a DNA sequence selected from the group consisting of:

(1) DNA encoding the amino acid sequence of a mammalian basic fibroblast growth factor (bFGF) polypeptide comprising the 146-amino acid residue sequence:

```
 1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-

 18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34
Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-

 35  36  37  38  39  40  41  42  43  44  45  46  47  48  49  50  51
His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-

 52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67  68
Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-

 69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85
Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-

 86  87  88  89  90  91  92  93  94  95  96  97  98  99 100 101 102
Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119
Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-

120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-

137 138 139 140 141 142 143 144 145 146
Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,
```

or the amino acid sequence in which 1 to 3 amino acids may be substituted in said amino acid sequence and which is biologically active as a fibroblast growth factor;

(2) DNA encoding the amino acid residue sequence of a bFGF

polypeptide which comprises an N-terminally extended analogue of the above 146-amino acid residue sequence and which is biologically active as a fibroblast growth factor; and

(3) DNA encoding an N-terminally shortened fragment of the above 146 amino acid reside sequence and which is biologically active as a fibroblast growth factor;

and which is capable of expressing the encoded bFGF polypeptide.

(Translation)

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Inventor(s):	ESCH, Frederick, Stephen BAIRD, Andrew BOHLEN, Peter GOSPODAROWICZ, Denis, Jersy LING, Nicholas, Chai-Kwan
Applicant:	THE SALK INSTITUTE FOR BIOLOGICAL STUDIES

Japanese claim(s) published:

Please refer to the attached sheet(s).